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First isolate of *Toxoplasma gondii* from arctic fox (*Vulpes lagopus*) from Svalbard

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Abstract

Cats are considered essential for the maintenance of *Toxoplasma gondii* in nature. However, *T. gondii* infection has been reported in arctic fox (*Vulpes lagopus*) from the Svalbard high arctic archipelago where felids are virtually absent. To identify the potential source of *T. gondii*, we attempted to isolate and genetically characterize the parasite from arctic foxes in Svalbard. Eleven foxes were trapped live in Grumant (78°11′N, 15°09′E), Svalbard, in September 2005 and 2006. One of the foxes was found to be seropositive to *T. gondii* by the modified agglutination test (MAT). The fox was euthanized and its heart and brain were bioassayed in mice for the isolation of *T. gondii*. All 10 mice inoculated with brain tissue and one of the five inoculated with heart developed MAT antibodies, and tissue cysts were found in the brains of seropositive mice. Two cats fed tissues from infected mice shed *T. gondii* oocysts. Genotyping using 10 PCR-RFLP markers and DNA sequencing of gene loci *BSR4*, *GRA6*, *UPRT1* and *UPRT2* determined the isolate to be Type II strain, the predominant *T. gondii* lineage in the world.
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1. Introduction

The coccidian parasite *Toxoplasma gondii* has a global distribution. The domestic cat and other felids are the only definitive hosts known (Frenkel et al., 1970), in which the sexual parasite cycle takes place in the intestines and oocysts are produced (Dubey et al., 1970). In intermediate hosts, which comprise virtually all warm-blooded animals, the parasite multiplies asexually in various tissues, resulting in formation of tachyzoites and tissue cysts containing bradyzoites (Dubey and Beattie,

1988). Animals and humans can become infected by intake of food or water contaminated with oocysts, by ingestion of infected tissues, or transplacentally (Dubey and Beattie, 1988). Cats are considered essential in the life cycle of *T. gondii*; infection was either absent or of very low frequency in animals on isolated islands without cats (Wallace, 1973; Munday, 1972; Dubey et al., 1997).

The high arctic archipelago of Svalbard (78°–81°N, 10°–30°E) is located midway between the Norwegian mainland and the North pole. The arctic fox (*Vulpes lagopus*, formerly *Alopex lagopus*) is a top predator feeding both from the marine and the terrestrial food chain, and an opportunistic feeder and scavenger. Major food sources are migratory birds, arriving in the spring every year, and Svalbard reindeer (*Rangifer tarandus*

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platyrhyncus) carcasses (Prestrud, 1992; Eide et al., 2005).

Three arctic foxes found dead on Svalbard in 2000 were previously diagnosed with disseminated toxoplasmosis (Sørensen et al., 2005). Antibodies against *T. gondii* were recently reported in mammal and bird species from Svalbard, including arctic fox (Prestrud et al., 2007). Domestic cats are prohibited on Svalbard, and there are no wild felids present. However, a very limited and stationary cat population exists in the village Barentsburg (78°5′N, 14°15′E). The harsh arctic climate does not allow the cats to roam outside the village. The results in Prestrud et al. (2007) gave reason to believe that oocysts are of minimal importance for transmission of *T. gondii* in the Svalbard ecosystem, because the terrestrial herbivores studied (reindeer and sibling voles, *Microtus rossiaemeridionalis*) were all seronegative.

Aim of the present study was to isolate *T. gondii* from this virtually cat free, high arctic area. The arctic fox population on Svalbard is closely monitored by the authorities (http://mosj.npolar.no/; Fuglei et al., 2003), and obtaining fresh tissues from a seropositive fox was a great challenge. For the present study, permission from the Governor of Svalbard and the Norwegian Animal Research Authority was sought to study *T. gondii* infection in arctic foxes.

2. Materials and methods

2.1. Foxes and assaying for T. gondii

A total of 11 wild arctic foxes were trapped live (Tomahawk Live Trap, 100 cm × 30 cm × 30 cm) in September 2005 ($n = 5$) and 2006 ($n = 6$) in Grumant (78°11′N, 15°09′E), Svalbard. All were less than 6 months old. A blood sample (0.5 ml) was obtained from the cephalic vein. The sample was allowed to clot, and serum was collected and assayed for antibodies (IgG) against *T. gondii* at twofold dilutions of 1:40 to 1:1280 using the direct agglutination test (DAT; Toxoscreen DA kit, bioMerieux S.A., Marcy-l′Etoile, France), performed in a heated tent (12–15 °C). The foxes were caged individually (60 cm × 46.5 cm × 54 cm metal wire dog cage) for 6–8 h during the analysis.

One of the 11 foxes, a female weighing 2 kg, had a positive DAT at serum dilutions up to 1:1280. The fox was anesthetized by intramuscular injection of a mixture of 0.5 ml (12.5 mg/kg body mass) ketamin (Ketalar® 50 mg/ml, Pfizer Inc., NY) and 0.5 ml (0.25 mg/kg) medetomidine (Domitor® 1 mg/ml, Orion

Corp., Turku, Finland). This is five times the recommended dose for immobilization/anaesthesia of arctic fox (Fuglei et al., 2002; Kreeger et al., 2002). Surgical anaesthesia was induced within seconds. Blood was collected by cardiac puncture, the fox was killed by cervical dislocation, and the brain, heart and serum were kept cool but unfrozen during transportation via Norway to the Animal Parasitic Diseases Laboratory (APDL), U.S. Department of Agriculture (USDA), Beltsville, MD. Total transportation time was 16 days. The serum was retested for *T. gondii* antibodies by the modified agglutination test (MAT) as described previously (Dubey and Desmonts, 1987). The fox had a positive MAT in 1:1600 dilution; the MAT and DAT are essentially similar.

2.2. Bioassay in mice

The brain and heart were separately homogenized in a Waring® blender. The heart homogenate and half of the brain homogenate were separately digested with pepsin (Dubey, 1998), the rest of the brain homogenate was kept undigested and these three volumes of processed tissue were divided into 1 ml dosages and injected subcutaneously into three groups of five Swiss Webster (SW) outbred albino mice per group (Taconic Farms, German Town, NY).

On day 29 post-inoculation (p.i.), a blood sample was taken from each mouse and the sera were assayed by MAT at 1:25 dilution. The mice were killed by atlanto-axial dislocation after 32–60 days p.i. To verify *T. gondii* infection, brain smears were made from each mouse and examined for tissue cysts by microscopy (Dubey and Beattie, 1988).

2.3. Bioassay in cats

Skinned and eviscerated carcasses from *T. gondii* infected mice were minced and fed to two *T. gondii* seronegative cats (Dubey, 1995). The mouse brains were homogenized separately with mortar and pestle and mixed with 0.5 ml of saline per brain, and then fed to the cats using a 5 ml syringe. Faecal samples from both cats from days 3–5 were pooled prior to oocyst examination, as were samples from days 6–9. The pooled samples were examined for presence of *T. gondii* oocysts by sucrose flotation and microscopy from days 2–9 after feeding, and when oocysts appeared, they were collected by sucrose flotation and water sedimentation, and sporulated in 2% sulphuric acid (Dubey, 2006).

Table 1
Infectivity and pathogenicity of *T. gondii* oocysts of the arctic fox isolate to mice

No. of oocysts administered	No. of mice dead (day of death)	No. of mice infected[a]
100,000 (10^{-1})	3 (10, 10, 12)	5
10,000 (10^{-2})	2 (7, 8)	5
1,000 (10^{-3})	0	5
100 (10^{-4})	0	5
10 (10^{-5})	0	4
1 (10^{-6})	0	1
<1 (10^{-7})	0	0

[a] Five mice were fed oocysts from each dilution.

2.4. Pathogenicity of oocysts of the fox isolate to mice

Oocysts were counted in a hemocytometer, neutralized with sodium bicarbonate (final pH 7.2), and diluted 10-fold until oocysts were no longer observed (10^{-7} dilution), starting with 1.7×10^6 oocysts/ml. An aliquot (0.5 ml/mouse) was inoculated orally in five female SW mice for each dilution (Table 1).

2.5. Genotyping

Mice were inoculated intraperitoneally with neutralized sporulated oocysts. Seven days after injection, tachyzoites were collected from mouse peritoneal lavage and inoculated into cultures of human fibroblast (HFF) cells in T25 flasks (Corning Inc., Acton, MA). After the parasites had lysed HFF cells, tachyzoites were harvested by filtering through a 3 µm PCTE filter membrane (Fisher, Hanouver Park, IL) and centrifugation (800 × *g*, 10 min). The pellets were resuspended in 1 ml of PBS (pH 7.2), and proteinase K (10 mg/ml; 20 µl) was added. The mixtures were incubated at 55 °C for 2 h to lyse the parasites and release DNA. Proteinase K was then inactivated at 95 °C for 15 min. The DNA lysates contained approximately 10^7 copies of genomic DNA per ml. Genotyping was performed at 10 genetic loci including *SAG1*, *SAG2*, *SAG3*, *BTUB*, *GRA6*, *c22-8*, *c29-2*, *L358*, *PK1* and Apico by a published protocol (Su et al., 2006).

For DNA sequencing of *BSR4*, *GRA6*, *UPRT1* and *UPRT2*, each target sequence was amplified by PCR and then the PCR products were purified and sequenced from both ends by sequencing primers (Table 2).

3. Results

All mice inoculated with fox brain material and one of the five mice inoculated with digested heart developed antibodies against *T. gondii*, but remained asymptomatic. Tissue cysts were found in the brains of all 11 seropositive mice when killed on days 42 (*n* = 1), 44 (*n* = 2) or 60 (*n* = 8) p.i. The two cats fed infected mice shed *T. gondii* oocysts starting 3–6 days p.i.

Infectivity data indicated that 100,000 infective oocysts were present in the 10^{-1} suspension of oocysts fed to mice (Table 1). Five of the 10 mice fed 10,000 or 100,000 oocysts died of acute toxoplasmosis and tachyzoites were found in their mesenteric lymph nodes or lungs (Table 1).

Genotyping at 10 genetic loci including *SAG1*, *SAG2*, *SAG3*, *BTUB*, *GRA6*, *c22-8*, *c29-2*, *L358*, *PK1* and *Apico* indicated that the fox isolate was identical to the Type II strain Me49 (Suzuki et al., 1989). Analysis of DNA sequences of genes *BSR4* and *GRA6*, as well as two introns (*UPRT1* and *UPRT2*) of the UPRT gene further confirmed that the fox isolate was identical to the Me49 strain.

4. Discussion

Many factors including the age, gender, host species, and the stage and genotype of *T. gondii* ingested can

Table 2
Primers used for PCR and sequencing of *T. gondii* from arctic fox

GRA6 (Fazaeli et al., 2000 and this study)	PCR primers: GRA6-For GTAGCGTGCTTGTTGGCGAC; GRA6-Rev TACAAGACATAGAGTGCCCC
	Sequencing primers: GRA6-SqF GTGGACAGCAAGAAGCAGTG; GRA6-SqR GCACCTTCGCTTGTGGTT
BSR4 (GenBank accession number for Type II strain PRU: AF394604)	PCR primers: BSR4-F CAAGGCTAACCGTAGCGGGT; BSR4-R CTCTTCAGGAACTTGGGGAA
	Sequencing primers: BSR4-SqF GGCACAGAAGTAACGCTGAA; BSR4-SqR CTCCGTTGACATCTCCACCT
UPRT1 (Su et al., 2003)	PCR primers: UPRT-F1 CCCGATATTCGACAAACGAC; UPRT-R1 GAGCCGTCTGCTTCATGAGC
	Sequencing primers: UPRT-sqF1 TCAACCGAAGTTTGCTTTCC; UPRT-sqR1 CCCTGAAAGGAAGCACGTAA
UPRT2 (Su et al., 2003)	PCR primers: UPRT-F2 TCTTGTTTGCTTTCCTCGGC; UPRT-R2 CGGTCAGCGGTCTGTCAAAA
	Sequencing primers: UPRT-sqF2 CTGCGACGACATACTGGAGA; UPRT-sqR2 CATAAAGGGTTGGCTGCATT

affect the clinical outcome of infection. Until recently, *T. gondii* isolates were grouped as mouse virulent or avirulent. Type I strains were considered mouse virulent and Type II and Type III strains were regarded as mouse avirulent. However, there is no evidence that mouse virulence is correlated with virulence of *T. gondii* for higher animals (Dubey, 2006). Little is known about the genetic makeup or mouse virulence of the *T. gondii* isolates from arctic foxes. To the best of our knowledge *T. gondii* has previously been typed from a single red fox (*Vulpes vulpes*) and a grey fox (*Urocyon cinereoargenteus*) (genotyping based on PCR-RFLP using only one marker), and both of these isolates from USA were *SAG2* Type II and they were not virulent for mice (Dubey et al., 2004a). In this respect the arctic fox isolate in the present study is similar to the previous fox isolates. Experimentally, red foxes fed *T. gondii* oocysts or tissue cysts remained asymptomatic (Dubey, 1983; Dubey et al., 1999). However, pups born by three arctic foxes that had been inoculated parenterally with a highly virulent *T. gondii* 10–16 days before delivery died of acute toxoplasmosis (Bjerkås, 1990). In general, fatal toxoplasmosis is often associated with immuno-suppression (Davidson et al., 1993; Minkoff et al., 1997; Nissapatorn et al., 2004). Why three arctic foxes on Svalbard died of acute toxoplasmosis in 2000 is unexplained, because only one of them had a co-infection that can be linked to immunosuppression (*Yersinia pseudotuberculosis* and *Salmonella enteritidis*; Sørensen et al., 2005).

Genotyping at 10 genetic loci and DNA sequencing of *BSR4*, *GRA6*, *UPRT1* and *UPRT2* of the *T. gondii* isolate from the arctic fox in this study showed that the isolate was identical to the typical Type II strains, the predominant lineage in Europe. The barnacle goose (*Branta leucopsis*) and the pink-footed goose (*Anser brachyrhyncus*) are migratory species that winter in Britain and continental Europe, respectively (Owen and Gullestad, 1984; Madsen et al., 1997). Both are abundant on Svalbard during summer (Madsen et al., 1997; Strøm, 2006) and geese are known to be an important food source to the arctic fox (Fuglei et al., 2003; Eide et al., 2005). Antibodies against *T. gondii* have been demonstrated in barnacle geese from Svalbard (7%, $n = 149$; Prestrud et al., 2007), hence, migratory birds may be major reservoirs for transmission of *T. gondii* from the European continent to Svalbard. Viable *T. gondii* has previously been isolated from Canada goose (*Branta canadensis*) and domestic goose (*Anser anser*) from USA (Dubey et al., 2004b, 2007).

One single isolate is not sufficient to draw definitive conclusions with regards to strains present in the ecosystem, but this first isolate clearly shows that if an arctic strain exists, it is not the only one present on Svalbard. Therefore, an earlier hypothesis of a specific arctic strain of *T. gondii* in this virtually cat-free ecosystem is not supported by this study. Further studies of *T. gondii* from mammals and birds on Svalbard are necessary to determine diversity and epidemiology of the parasite.

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